DIFFERENTIAL GENE EXPRESSION DURING ADIPOGENESIS IN CULTURED BOVINE ADIPOCYTES

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2006

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
Master of Science
May, 2012

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

The distribution and amount of fat deposition d in an animal greatly influences palatability, eating quality of beef, tenderness, feed efficiency and meat price. Marbling is the deposition of fat in the intramuscular depots. It enhances tenderness and palatability of beef product; hence it influences the quality and economic return of beef carcass (Sainz, 2000; Cheong et al., 2009; Mizouchi et al., 2010; Pickworth et al., 2011). Subcutaneous fat deposits on the other hand are undesirable due to obvious health issues it causes in human and hence cause great economic loss. Selective breeding, nutrition management and hormone supplementation are some techniques used to produce animals with higher amount of marbling. Recently, efficient molecular techniques have become available to identify genetic markers that define marbling in an animal. The objective of this study is to identify the genes regulating intramuscular and subcutaneous fat deposition in beef cattle that may help in manipulation of site and rate of fat deposition.

A breeding program to produce quality beef products at a competitive cost is one of the greatest challenges in the beef industry (Tan et al., 2006). Value of beef products is defined by its quality and one of the major attributes of quality is fat content. Insufficient amount of intramuscular (IM) fat and excess amount of subcutaneous (SC) fat deposits in beef cattle not only effects the product quality but also impose a great degree of economic impact on production (Grant et al., 2008a; Grant et al., 2008b; Taniguchi et al., 2008a). A study by Novakofski (2004) showed that the cost of fat production is greater than the cost for muscle production. Therefore the location of fat deposition becomes important for the producers. Intramuscular fat deposits known as marbling are a desirable trait for producers because it increases the palatability and tenderness of beef (Field et al., 1966; Wang et al., 2008; Nishimura et al., 2010). In a study done by Nishimura et.al (2010) using scanning electron micrography, adipose tissue was shown to be formed between muscle fiber bundles. Due to this, the honeycomb structure of endomysia is partially broken and separates the perimysium into thin collagen fibers in *longissimus* muscles compared to *semitendinosus* muscles with lower crude fat content (Nishimura et al., 1999; Nishimura et al., 2010). Quality of beef is generally determined by the marbling, color, firmness and texture of meat (Hwang et al., 2010) and USDA grading system is mainly based on marbling and gives the best grade to carcasses with higher marbling (Savell et al., 1987). Consumers have different preference for marbling in beef; the current trend is towards lean, fat free meat products. In recent times, with the change in trends of consumer preference to a leaner beef product, there is growing demand to

revise the grading system to better reflect the market trend (Savell et al., 1987). In a study done by Killinger et al.(2004), Chicago and San Francisco consumers who demand lean beef were willing to pay more for lean meat (Killinger et al., 2004; Behrends et al., 2005). Subcutaneous fat on other hand is always an undesirable trait, and is discarded causing an estimated $4.41 billion loss with 1.99 billion on production and 2.42 billion on trimming it from the carcass (Smith, 1991). A study by Schmidt et al. (2002) places the net loss per animal due to trimming of excess subcutaneous fat at $69.12 for steers and $61.12 for heifers.

THE ADIPOSE ORGAN

The adipose organ is a multi-depot complex and diffuse organ containing adipose tissue (Cinti, 2005). It is different from the chemical fat in the sense that it consists of different types of cells, mainly adipocytes containing lipid, whereas the latter is just the lipid that can be extracted using an organic solvent (Allen, 1976). There are two distinct types of adipose tissue, the white adipose tissue (WAT) and the brown adipose tissue (BAT) (Cinti, 2001).

Brown Adipose Tissue (BAT)

The brown adipose tissue (BAT) is found in new born humans and in intrascapular spaces of small mammals (Gesta et al., 2007; Wolf, 2009). Brown adipose tissue is also present in hibernating animals functioning to provide heat by means of nonshivering thermogenesis (Yan et al., 2006). BAT can be found in adult small mammals like rodents but in humans it is replaced by the white adipose tissue (WAT) with few BAT interspersed within it. BAT cells consist of cytoplasm with a central nucleus and contain numerous lipid droplets and mitochondria giving it the namesake brown color. BAT is highly vascularized compared to WAT. The main function of BAT is thermogenesis; it oxidizes fat and transmits the energy thus produced as heat (Barbara et. al, 2003; Lehr et al, 2009; Wolf, 2009; Shabalina et al, 2010). The mitochondria in BAT contain uncoupling protein 1 (UCP1) in their inner membrane. UCP1 is a proton transporter, facilitating the transfer of anions from the inner to the outer mitochondrial membrane and the transfer of protons from the outer to the inner mitochondrial membrane and causes the loss of electrochemical gradient which the mitochondria use to produce ATP. Thus UCP 1 separates oxidative phosphorylation from ATP synthesis and the energy is dissipated in the form of heat (Adams, 2008; Wolf, 2009).

White Adipose Tissue (WAT)

White adipose tissue is the main lipid store, composed of spherical adipocytes. White adipose tissue cells are spherical with a rim of cytoplasm, and a large membrane enclosed lipid droplet which pushes the nucleus to the margin of the cell. White adipose tissue contains fewer mitochondria and is less vascularized than BAT. The main function of WAT is to store energy in the form of lipid (Mohamed-Ali et al., 1998; Ntambi et al., 2000; Trayhurn et al., 2001; Karolina et al, 2011). When there is a demand for energy, WAT releases fatty acids by the breakdown of triglycerides and releasing them into circulating blood which is used for energy production in muscles (Singh et al., 2009). That is just one of many functions of WAT which also functions in thermal insulation in case of marine animals like the blubber in whales and seals and serves a metabolic function in glucose homeostasis. WAT also plays a role in inflammatory process with preadipocytes acting like macrophages (Cousin et al., 1999). The study by Trayhurn et al. (2001) also sheds light on adipose tissue as an endocrine and secretory organ. White Adipocytes secrete leptin, a hormone important in energy balance, in addition to other proteins such as angiotensinogen, adipsin, acylation-stimulating protein, retinol binding protein and tumor necrosis factor. These proteins are involved in lipid metabolism, vascular hemostasis, the complement system and some are inflammatory cytokines thus establishing WAT as a major secretory and endocrine organ (Mohamed-Ali et al., 1998; Trayhurn et al., 2001; Ahima, 2006; Chen et al., 2010; Lagowska et al., 2011). In this review we will be focusing more on the location of WAT as it is one of the major

determinants of meat quality and economic return in beef cattle. The several depots of adipose tissue that we are interested in beef cattle are subcutaneous, visceral and intramuscular depots (Klien, 2004; Sainz et al., 2000; Dodson et al., 2010). The visceral fat, also known as intraperitonial fat, is composed of omental, mesenteric and retroperitoneal fat and is located alongside the ventral surface of the kidney and the dorsal borderline of the intestines (Mårin et al., 1992; Wajchenberg, 2000). Subcutaneous fat is located just under the skin or hide in case of animals. Subcutaneous fat develops in three layers, the outer layer develops first and acts as insulation for animals followed by the middle layer which is the thickest and has high metabolic activity. The third layer develops last and is very thin (Gerrard et al., 2003). Intramuscular fat, also known as seam fat, is located between the individual muscles or group of muscles (Gerrard et al., 2003) and is the fat depot we are interested in.

ADIPOGENESIS

Adipogenesis is defined as a coordinated proliferation, differentiation and initial accumulation of lipid droplet in cells (Grant et al., 2008b; Taniguchi et al., 2008a). It is a complex process influenced by genetics, environment, breed, age, sex, nutrition and management (Nakachi et al., 2008; Pyatt et al., 2005). Adipose tissue in mammalian embryos arise relatively late in the developmental pathway from pluripotent mesenchymal cell population (MacDougald and Lane, 1995). Mesenchymal progenitor

cells are also present in the stroma of bone marrow and adipose tissue of adult mammals (Dennis et al., 1999). While it was suggested that the number of adipocytes remain constant after birth, new findings have revealed otherwise. It has now been established that adipogenesis occurs throughout lifetime as a result of cell turnover and increased fat mass for storage of excess calories (Gregoire et al., 1998; Rosen and MacDougald, 2006).

The process of adipocyte differentiation has been extensively studied using in vitro models and is considered to closely mimic the differentiation process in vivo (Gregoire et al., 1998; Rosen and Spiegelman, 2000). Adipocyte differentiation has been studied using the preadipose cell line and the primary preadipocytes. The most extensively used preadipose cell lines are 3-day transfer, inoculum 3 x 10^5(3T3) cells lines; 3T3-F442A and 3T3-L1 cell lines, which were created from clonally isolated Swiss 3T3 cells from 17-19 day mouse embryos (Gregoire et al., 1998). These cells are morphologically similar to fibroblasts but they are already committed to adipogenesis (Rosen and Spiegelman, 2000). Other cell lines that are used for studying adipocyte differentiation in vitro are ES cells, TA1, Ob17 and CH3 10 T1/2 (Gregoire et al., 1998). Primary preadipocytes have been successfully isolated and cultured from various species, and possess certain advantage over the preadipose cell lines. First the primary preadipocytes are diploid and thus will represent the in vivo differentiation process better that the aneuploid cell lines. Second, primary preadipocytes can be obtained from different adipose depots and from different stages of development, thus making it more preferable

to study molecular and biochemical differences at various sites of adipose depots. Primary preadipocytes do have a few drawbacks such as a potential cellular heterogeneity and the donor dependent differentiation capacity which significantly decreases with the donor's age (Gregoire et al., 1998). The process of adipocyte differentiation can be divided into five phases: determination, growth arrest, mitotic clonal expansion, early change in gene expression and terminal differentiation (MacDougald and Lane, 1995; Rosen and MacDougald, 2006)

Determination

This phase is characterized by the commitment of the pluripotent stem cells to adipocyte lineage. At this point, the stem cells are morphologically indistinguishable from its precursor but will have lost its ability to differentiate into any other cell type (Rosen and MacDougald, 2006).

Growth arrest

The next phase in cell differentiation is growth arrest. Cells reach confluency and demonstrate contact inhibition; at this stage the cells undergo growth arrest at the G_o/G_1 cell cycle. Transcription factors CEBPα and PPARγ are involved in the process of growth arrest (Gregoire et al., 1998; Zhu et al., 2009). CEBPα increases the level of p21/SDI-1

mRNA, which has been found to be highly elevated in growth arrested HT1 cells in a study conducted by Timchenko et al (1996). The study also suggested that during this phase CEBPα maintains an increased level of p21/SDI-1 protein in two ways; first by increasing the expression of p21/SDI-1 gene and second by stabilizing the p21/SDI-1 protein. PPARγ co-operates with CEBPα to induce growth arrest, it has also been established that PPARγ alone is sufficient for induction of growth arrest. Its expression is found in concurrence with the decrease in DNA binding activity of E2f/DP, a transcriptional regulator of many genes involved in cell growth (Gregoire et al., 1998; Rosen and Spiegelman, 2000).

Mitotic clonal expansion

For cells at the stage of growth arrest to continue through the process of differentiation, they need an optimum combination of mitogenic and adipogenic signals. In a study with 3T3-L1 cells, the growth arrested 3T3-L1 preadipocytes were found to first undergo mitotic clonal expansion followed by expression of adipogenic genes (Tang et al.., 2003). Growth related proteins like retinoblastoma proteins pRB, p107 and p130 shows change in expression during 3T3-L1 differentiation, with p107 showing a transient increase in expression specific to clonal expansion. These proteins bind to the E2F/DP complex which inactivates growth-promoting transcriptional activities (Gregoire et al., 1998). Also, Gas-6 gene, a member of a group of growth arrest-specific (gas) genes, is expressed

during clonal expansion in post confluent preadipocytes. On the other hand only Gas-1 and Gas-3 are expressed during pre-confluent proliferation of cells. It has also been suggested that a phosphorylation-dephosphorylation mechanism is involved in clonal expansion. There is an increase in tyrosine phosphatase HA2, which blocks adipocyte differentiation during clonal expansion but not during later phases. Study using phosphatase inhibitor vanadate has shown to overcome the effect of HA2 hence establishing the fact that phosphorylation-dephosphorylation mechanism is also involved in clonal expansion (Liao and Lane, 1995).

Early changes in Gene Expression

The earliest sign of adipocyte differentiation is suggested to be the expression of lipoprotein lipase (Gregoire et al., 1998). CEBPα expression is induced in the cells post mitotic clonal expansion. CEBPα expression leads to the activation of several adipogenic genes like, 422/aP2, Glut4 and SCD1 gene. Several other genes that are differentially expressed at the stage of terminal differentiation have been identified (MacDougald and Lane, 1995).

Terminal differentiation

Terminal differentiation is characterized by the loss of the cell's ability to dedifferentiate. This terminally differentiated state is maintained by the expression of CEBPα. CEBPα operates by blocking mitosis and by tans-activation of other adipogenic genes (MacDougald and Lane, 1995). At this point in adipocyte differentiation, the cells exhibit de novo lipogenesis and develop sensitivity to insulin (Gregoire et al., 1998).

TRANSCRIPTIONAL REGULATIONS OF ADIPOGENESIS

Several transcription factors that regulate adipogenesis have been identified. They act cooperatively and sequentially leading to commitment and differentiation of stem cells to adipose lineages (Mandrup et al., 1997). These factors include peroxisome proliferator activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), insulin, insulin like growth factors (IGF), myostatin, cAMP, glucocorticoids, triiodothyronine, growth hormone (GH) , and prostaglandin, tumor necrosis factor- β (TGF-β), tumor necrosis factor-α (TNFα), leptin, resistin, macrophage colony stimulating factor (MCSF), angiotensin II, autotoxin lysophosphatic acid (ATX-LPA) etc. (Ailhaud et al., 1992; Kim et al., 1996; Kershaw et al., 2004; Cinti., 2005; Hirai et al., 2007). A combination of insulin, cAMP, fatty acid and glucocorticoids is considered necessary for the differentiation of preadipocyte in cell culture system (MacDougald and Lane, 1995). The

progenitor cells can be hormonally induced once they are confluent, resulting in a mitotic clonal expansion, growth arrest and finally differentiation (Mandrup et al., 1997).

Peroxisome proliferator activated receptor γ (PPARγ)

Peroxisome proliferator activated receptor γ is a member of nuclear hormone receptor family primarily expressed in adipose tissue (Chung et al., 2008; Shockley et al., 2009) and was the first member identified in its family (Mandrup et al., 1997). It is considered necessary and sufficient for adipogenesis, hence it is the 'master regulator' of adipogenesis (Sadowski et al., 1992; Rosen and Spiegelman, 2000; Rosen and MacDougald, 2006; Shockley et al., 2009). Ectopic expression of PPARγ has shown to be able to convert fibroblasts into differentiation competent preadipocytes (Tontonoz et al., 1994). It is not only important for the induction of adipogenesis but also for the maintenance of the differentiated state (Rosen and MacDougald, 2006). At initiation of adipogenesis CEBPα is induced which binds to the promoter of PPARγ to induce its expression (Du.M et al., 2009). PPARγ forms a heterodimer with the retinoid X receptor (RXR) and induces differentiation (Chung et al., 2008; Du.M et al, 2009). PPARγ also promotes the expression of CEBPα creating a self-reinforcing regulatory loop (Du.M et al., 2009). The role of PPARγ has been established by gain-of function studies and confirmed by gene deletion experiments (Mandrup et al., 1997; Rosen and Spiegelman, 2000; Rosen and MacDougald , 2006). PPARγ occurs in two isoforms PPARγ1 and PPARγ2. PPARγ1 is expressed in many tissues but PPARγ2 is more specific to fat

tissue (Rosen and Spiegelman, 2000). Although both isoforms are induced during adipogenesis, the relative role of each is not yet confirmed (Mandrup et al., 1997; Rosen and MacDougald, 2006). There are contrasting results about the relative role of PPARγ2 in adipogenesis. Rosen and MacDougald (2006) has pointed out that there are two contrasting results out for the relative role of PPARγ1. In one study conducted by Ren et al. (2002) ectopic expression of PPARγ2 induces adipogenesis whereas PPARγ1 fails to do so, when endogenous PPARγ1 and PPARγ2 promoter have been inhibited. A contrasting result has been out forward by Mueller et al (2002) where both isomers were able to promote adipogenesis in a $Pparg^{-/-}$ fibroblasts. In other in vivo studies using Pparg2- knockout mice, one showed a decreased adipose tissue and the other showed normal adipose tissue but developed insulin resistance. Hence it shows that PPARγ2 is not as important for adipogenesis in vivo (Rosen and MacDougald, 2006; Nakachi, 2008). PPARγ activation is ligand dependent. Inactive PPARγ is associated with it co-repressors, which silences its transcriptional activity. Ligands bind to PPARγ replacing the co-repressors with co-activators. The co-activators have histone acetyl transferase activity, which leads to chromatin condensation and gene expression (Du.M et al, 2009).

CAAT/enhancer binding proteins(C/EBPs)

C/EBPs are members of the basic leucine zipper family transcription factors. They can act either as homo or heterodimer (Kim et al., 1996; Olofsson et al. 2008). Members of

the C/EBP family were the ones first identified to play a major role in adipogenesis (Gregoire et al., 1998), and are expressed in a cascade during adipogenesis (Rosen and Spiegelman, 2000; Rosen and MacDougald, 2006). C/EBPβ and C/EBPδ are induced first leading to the induction of C/EBPα (Rosen et al., 2006). Expression of C/EBPβ and C/EBPδ also leads to the expression of PPARγ; while the expression of C/EBPα occurs right before the end product of the fat genes occur (Rosen and Spiegelman, 2000). It has been established that even though the C/EBP are important for adipogenesis they cannot function in absence of PPARγ. In the absence of PPARγ, C/EBPβ fails to induce the expression of C/EBPα, as the former is required for the release of histone deacetylase-1 from the C/EBPα promoter (Rosen and MacDougald, 2006). Thus PPARγ promotes C/EBPα and forms a self-regulatory loop (Du.M et al., 2009). C/EBPα is a member of the C/EBP family and an important transcriptional factor in activation of adipogenesis (Mandrup et al., 1997; Olofsson et al., 2008) and is responsible for insulin sensitivity (Rosen and MacDougald, 2006). Though C/EBPα is induced during adipogenesis it is not adipose-specific in vivo (Kim et al., 1996). The importance of C/EBPα for adipogenesis has also been reinforced by the absence of adipogenesis using antisense C/EBPα RNA in 3T3-L1cells (Samuelson et al., 1991; Umek et al., 1991; Lin et al., 1992; Mandrup et al., 1997). C/EBPα is expressed just before the initiation of transcription of most adipogenic genes, by binding and transactivating the promoter of the genes (Kaestner et al., 1990; Park et al., 1990; Mandrup et al., 1997; Ramji., 2002). C/EBPα shows anti-mitotic activity, which terminates the mitotic clonal activity. The termination of mitotic clonal

activity coincides with the expression of C/EBPα and the activation of adipogenic genes (Mandrup et al., 1997; Olofsson et al. 2008). C/EBPα is also suggested to maintain the adipocytes phenotype through auto activation of its own gene (Mandrup et al., 1997; Olofsson et al. 2008). A study by Yeh et al. (1995) shows that C/EBPβ also promotes differentiation. C/EBPβ is induced during early stage of differentiation and in turn induces the expression of PPARγ (Wu et al., 1995).

Sterol regulatory element binding proteins (SERBP)

SREBPs are membrane bound transcription factors and functions as regulators of cholesterol and in fatty acids metabolism. SREBPs facilitate the transcription of more than 30 genes that are involved in the synthesis and uptake of fatty acids, triglycerides and cholesterol (Horton et al., 2003; Hao et al., 2010). SREBPs are sequestered in the endoplasmic reticulum (ER) in the presence of cholesterol, in the absence of which SREBPs undergo a proteolytic transformation which activates sets of target genes controlling lipid metabolism (Espenshade, 2006). SREBP occurs in three subtypes, SREBP-1a, SREBP-1c and SREBP-2. SREBP-2 is ubiquitously present and regulates cholesterol metabolism. SREBP-1 is more abundantly present in liver and adrenal gland regulates fatty acid and triglyceride metabolism. SREBP-1a and SREBP-1c are produced by alternate splicing. SREBP-1c has a weaker transcriptional activity than SREBP-1a because of its shorter N-terminal transactivation domain (Ryuichiro, 2010).

The N-terminal and C-terminal domain project into the cytoplasm and are linked by a membrane spanning domain. The N-terminal domain is a basic helix-loop-helix leucine zipper and the C-terminus forms a tight complex with the SREPB cleavage activating protein (Scap) which is a sterol sensor (Horton, 2002; Ryuichiro, 2010). SREBP is activated by insulin and induced in the initial stage of adipocyte differentiation and studies have shown that it up regulates the expression of PPARγ by producing an unknown endogenous ligand by binding to the response element of PPARγ1 and PPARγ3 promoters (Kim et al., 1998; Fajas et al., 1999; Inoue et al., 2001; Commerford et al., 2004; Du et al., 2010). SERBP-1c also plays a key role in expression of 6-Phosphodehydrogenase (6PGDH). 6PGDH is an enzyme in the pentose phosphate pathways and is one of the main sources of nicotinamide adenine dinucleotide phosphate (NADPH) which is the reducing agent required for many biological processes; one of which is elongation of fatty acids (Rho et al., 2005). SERBP-1c also induces PPARγ coactivator (PCG1α) in BAT. PCG1α plays important role in transcriptional regulation and adaptive thermogenesis (Puigserver et al., 1998; Hao et al., 2010).

INTRAMUSCULAR PREADIPOCYTE DEVELOPMENT

Intramuscular fat is the fat present between the muscle fiber bundles, within the muscle. Both muscle cells and adipogenic cells arise from the mesenchymal stem cells (MSCs).

MSCs are very abundant in the skeletal muscle, at the early stages of development and most of them develop into myogenic cell and a small fraction of it develop into adipogenic cell, which results intramuscular fat accumulation (Du.M et al., 2009). In ruminants adipogenesis is initiated during mid-gestation (Ganalingham et al., 2005; Mulhausler et al., 2007; Du.M et al., 2009). Intramuscular fat in cattle is also known as the taste fat and fat from other deposits in the carcass are known as waste fat. This is due to the fact that fats from other depots like the subcutaneous fat depots are trimmed off at the time of slaughter. Intramuscular fat is desirable as it enhances the juiciness, tenderness, taste and thus value of meat (Lehnert et al., 2006). There have been efforts to enhance intramuscular fat, but for each pound of intramuscular fat obtained there is a 10 pound increase in waste fat (H. Rouse, 2001).

Environment, nutrition and genetics factors regulate adipogenesis through their effect on key signaling pathways that regulate adipogenesis in skeletal muscle. Maternal nutrients are an important factor during mid to late gestation period as it regulates the number of MSCs that are committed to adipogenesis in the fetus (Du.M et al., 2009). In a study done by Tong et al. (2009) over-nourishment of pregnant ewes by feeding 1.5 times the National Research Council (NRC) nutrient requirement showed enhanced adipogenesis in the fetal skeletal muscle. Genetic makeup of the animal has a higher significance on the distribution and deposition of adipocytes. An animal can have a very suitable environment and a balanced diet but unless it has the right genetic makeup, marbling will not be higher compared to an animal on a regular diet and with the right genetic makeup

for high marbling (Wang et al., 2005). Such genetic factors influencing carcass quality and composition such as marbling, fat thickness, tenderness, and flavor have a moderate to high degree of heritability. It is also strongly suggested that the metabolic function of fat is depot specific and that under conditions of energy depletion in the body, internal fat depots are more mobilized that subcutaneous fat depots (Hitoshi.S, 2002; Bertile et al., 2004). The development of fat depots starts in utero, and continues throughout life (Wang et al., 2005; Wang et al., 2008). It has been suggested that the preadipocyte development is regulated differently in subcutaneous and intramuscular depots (H. Rouse, 2001; Hausman et al., 2007). An animal with the genetic predisposition to marbling will show fat deposition more in intra muscular depots that any other sites (Wang et al., 2005). Identification of genes regulating marbling can thus ensure a sustainable and profitable future for the beef industry (Lehnert et al., 2006).Various genetic markers have been identified from late nineties onwards that can assist selection and also gives the flexibility to evaluate and manipulate the genetic merit of animals (Lehnert et al., 2006; Burrow et al., 2001; Bindon., 2004). Most of the identified molecular markers on adipose tissue development have been drawn from rodent (3T3-L1) and human cell culture systems. Ruminants are physiologically and anatomically different from monogastric animals hence the relevance of monogastrics to bovine/livestock is yet not clear (Taniguchi et al., 2008a; Taniguchi et al., 2008b). In a study conducted by Mizoguchi et al., using clonal bovine intramuscular preadipocytes (BIP) has shown the gene expression pattern to be very different from the gene expression pattern in 3T3 cell lines. The master

regulators of 3T3-L1 cell differentiation, C/EBPα and PPARγ were not detected during differentiation in BIP cell lines. Similarly glucose transporter GLUT-4 was also not detected. On the other hand, C/EBPδ which is not upregulated during 3T3-L1 differentiation was upregulated in BIP cell lines (Mizoguchi et al., 2010). Interestingly, a study done by Allen et al in 1976 had already established the differences in gene ontology and regulation of adipose cellularity in pigs (Allen 1976; Hausman et al., 2007).

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CHAPTER II

DIFFERENTIAL GENE EXPRESSION ANALYSIS USING MICROARRAY

INTRODUCTION

Marbling is the deposition of fat in the intramuscular fat depots in beef cattle. It enhances tenderness and palatability of meat; hence it influences the quality and economic return of beef carcasses (Cheong et al., 2009; Mizoguchi et al., 2010; Pickworth et al., 2011; Sainz, 2000). Genetics has a significant effect on the distribution and deposition of fat in an animal. An animal can have a very suitable environment and a balanced diet but unless it has the right genetic makeup, marbling will not be higher compared to an animal on a regular diet and with the right genetic makeup for high marbling (Wang et al., 2005). Such genetic factors influencing carcass quality and composition such as marbling, fat thickness, tenderness, and flavor have a moderate to high degree of heritability. The development of fat depots start in utero, and continue throughout life (Wang et al., 2005; Wang et al.,2008). It has been suggested that the preadipocytes development is regulated differently in subcutaneous and intramuscular depots (Hausman et al., 2007). An animal

with the genetic predisposition to marbling will show more fat deposition in intra muscular depots that any other site (Wang et al., 2005). Identification of genes regulating marbling can thus ensure a sustainable and profitable future for the beef industry (Lehnert et al., 2006). Various genetic markers have been identified from late nineties onwards, that can assist selection and also gives the flexibility to evaluate and manipulate the genetic merit of animals (Bindon, 2004; Lehnert et al.,2006; Burrow et al.,2001). Most of the identified molecular markers on adipose tissue development have been drawn from rodent (3T3-L1) and human cell culture systems. Ruminants are physiologically and anatomically different from monogastric animals and the relevance of mongastric-based studies to bovine/livestock adipogenesis is yet not clear (Taniguchi et al., 2008a; Taniguchi et al., 2008b).Additionally early markers for development of subcutaneous and intramuscular preadipocyte differentiation has never been compared. Interestingly, a study done by Allen in 1976 had already established the differences in gene ontology and regulation of adipose cellularity in pigs. Until now there have been few studies on gene expression for the identification of molecular pathways of bovine preadipocyte differentiation. Additionally such studies were mainly performed to analyze the differential expression of a known adipogenic transcription factors like PPAR-γ and CEBP-α (Wu et al., 2000; Yamada et al., 2007). Bovine microarrays have been developed containing up to 24,000 distinct transcripts and has also been used in gene expression studies in bovine (Taniguchi et al., 2008a). Although breed specific differences in gene expression for marbling have been studied, yet there is only limited information on comparison of gene expression during preadipocyte differentiation

between cells from economically important adipose depots in bovine (Quinkler et al., 2006; Wang et al., 2008). Even though the morphological and cellular events appear to be identical in different depots of adipogenic tissues; the gene expression pattern and activated pathways are different (Grant et al., 2008; Hausman et al., 2007). Microarray technology allows obtaining massively parallel data and their analysis (Schena et al., 1995). It facilitates the investigation of global gene expression and provides a comprehensive assessment of gene expression levels (Wang et al.,2005, Schena et al.,1995; Hegde et al,2000).

MATERIALS AND METHODS

This study has 3 biological replicates and 2 technical replicates. Tissues were collected from wheat grass fed steers brought for slaughter at Oklahoma Food and Agricultural Products Research and Technology Center abattoir. Subcutaneous and intramuscular fat tissues were collected immediately after de-hiding of carcasses. Tissues were collected with sterilized instruments and maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 100IU/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin-B and 500µg/ml gentamycin (Transport media) at 4°C. The tissues were brought back to the lab where they were digested using DMEM containing 2mg/ml Type II collagenase, 4mg/ml BSA, 20mM HEPES, 100IU/ml penicillin, 100µg/ml

streptomycin, and 0.25 µg/ml amphotericin-B and 500µg/ml gentamycin (Digestion media) to isolate cells. Isolated cells were grown in growth media containing high glucose, Dulbecco's modified Eagle medium (DMEM; Hyclone, South Logan, UT) with 10% Fetal bovine Serum (FBS; Hyclone, South Logan, UT) until confluency and sub cultured and stored in liquid nitrogen.

Cells Culture and Differentiation

This primary cell line was used for RNA extraction for microarray analysis. The cells were plated at an concentration of $2.5x10^4$ cells/well, in a 6 well cell culture plate in growth media. The media was changed first at 24 hours and then at every 48 hours and the cells were grown till confluent. The confluent cells were induced to differentiate using Induction media I containing DMEM F12/HAM (Hyclone, South Logan, UT) with 10% FBS (Hyclone, South Logan, UT) in addition to 0.25µM Dexamethasone (Sigma, St.Louis,MO), 500 µM isobutyl Methyl Xanthine (IBMX; Sigma, St.Louis,MO), 300 µM water soluble Oleic acid (Sigma, St. Louis, MO), 10µM Troglitazone (Sigma, St. Louis, MO) and 10 µg/ml Insulin (Sigma, St. Louis, MO). Induction media was changed every 48 hours with Induction media II. Induction media II had the same composition as Induction media I minus Dexamethasone and IBMX. Accumulation of fat droplets was confirmed by staining the differentiated cell with Oil Red-O stain.

RNA Extraction

The cells were harvested at 0, 6, 12, 24, 48, 72hr and 8 days' time points after induction. Cells were detached using Trypsin and re-suspended in growth media. Total RNA was extracted using the RNAqueous-4PCR® column based kit (Ambion, Austin TX). The quality of extracted RNA was verified using gel electrophoresis and NanoDrop® 1000 spectrophotometer (NanoDrop products, Wilmington, DE).

Microarray

Labeling

The RNA harvested at '0' hr time point was used as the control for the experiment. Epicenter TargetAmp® 1-Round Aminoallyl-aRNA Amplification kit (Epicenter®, Madison, WI) was used to synthesize and label cDNA. This kit uses a linear amplification method to generate aminoallyl-labeled antisense RNA (aRNA). The first step consisted of cDNA transcription of the poly (A) RNA component of the total RNA using oligo dT primers and a reverse transcriptase (Superscript III RT, Invitrogen, Carlsbad, CA). First the Oligo dT primer was annealed to the RNA and then the transcription was carried out at 50º C for 30 min. Second strand cDNA synthesis was carried out using the RNA fragments as primer. The fragments were obtained by treating

the cDNA: RNA hybrid with RNase H. The reaction for second strand synthesis was carried out at 65º C for 10 min. The double stranded cDNA contains a T7 transcription promoter such that the subsequent in vitro transcription will generate anti-sense RNA (aRNA). In this transcription reaction the UTP nucleotides is partially substituted with 5-(3-Aminoallyl)-UTP and the reaction was carried out at 42º C for 4 h. The aminoallyl antisense-RNA (AA-aRNA) was purified using MinElute PCR purification kit (Qiagen, Valencia, CA). Concentration of the obtained AA-aRNA was determined using the NanoDrop 1000 spectrophotometer. Five µg of the AA-aRNA was aliquoted and dried using a Speedvac centrifuge at 45ºC, and the remaining AA-aRNA was stored at -80ºC. After drying, the pellet was dissolved in 5µl of 0.2M sodium carbonate buffer at pH 9. The control and test AA-aRNA was coupled at room temperature for 2 hrs with 5µl of Invitrogen's succinimidyl ester Alexa Fluor® 555 and Alexa Fluor® 647 Reactive Dyes respectively in separate tubes. The dye-labeled AA-aRNA was purified using MinElute PCR purification kit (Qiagen, Valencia, CA) and eluted with 25µl of DNase/RNase free water. The amount of dye incorporated in the AA-aRNA was determined for all samples to be around 58-85 nucleotides/dye using the NanoDrop spectrophotometer.

Hybridization

The labeled AA a-RNAs were hybridized to the bovine oligo array designed by the Bovine Oligonucleotide Microarray Consortium. The array consists of 24,000 probes

consisting of 16,846 EST probes supplemented with 703 predicted RefSeq genes, 5,943 reproductive tissue ESTs with a BGA but no protein alignment, and 504 +/- controls (Elsik et al. 2005). Reference design model was used for microarray analysis with RNA extracted at time point 0 being used as the reference point. Before hybridizing with the labeled AA a-RNAs the array was pre hybridized overnight at 42ºC in 1X BlockIt buffer(Arrayit Corporation, Sunnyvale, CA). The pre-hyb solution was washed off and the slides were dried using a slide centrifuge. For hybridizing 20µl each of the Alexa Fluor® 555 and Alexa Fluor® 647 labeled AA-aRNA were combined with 40µl of HybIt 2 Hybridization Solution(Arrayit Corporation, Sunnyvale, CA). The hybridization mix with the total volume of 80µl was then applied to the pre-hybridized slide covered with M series lifterslips (Thermo Fisher Scientific, Braunchweig, Germany). The array with the hybridization mix was incubated at 42ºC for 18 hrs. in a humidified hybridization cassette. Post hybridization wash was carried out in a 50 ml conical tube with 2X SSC/0.2 SDS which was preheated to 42ºC. The cover slip slides off in this process and further washes in 2X SSC/0.2SDS for 15 minutes at 42ºC, then with 2XSSC at RT for 15 min and then 0.2X SSC at RT for 15 min was carried out. The slide was then spin dried using a slide centrifuge and stored in light proof slide holders.

Image acquisition

A ScanArray® Express confocal laser scanner (Perkins Elmer,Boston, MA,USA) was used for image acquisition at the Oklahoma State University

Microarray Core Facilities (OMCF)**.** At the time of image acquisition the laser power and the photomultiplier tube (PMT) gain was adjusted to minimize variance between the channels. Images of each slide were saved for further processing and analysis.

Data analysis

Obtained images were analyzed and processed using GenePix® Pro 4.0 software (Axon Instruments, Union City, CA). Gene array list (GAL) file was used for spot to feature recognition. Features that were of poor quality or absent were flagged as bad and removed from further analysis. The three replicates were analyzed using GenePix Auto-processor (GPAP) which merges and compares the replicate hybridizations. The bias due to dyes within each array was normalized using local lowess pin-by-pin intensity dependent normalization. Quantile normalization was used to balance the effect of dye bias between the three arrays. Log_2 expression ratio (M value) was calculated for each gene where M value = $\log_2(F647\text{-}B647_{intensity}/ F555\text{-}B555_{intensity})$. The genes with a $\log_2$ ratio of >1 (up-regulated) or < -1 (down-regulated), i.e. a two-fold change, were selected to have significant difference in expression. Correlations among the three arrays were calculated using the Spearman rank correlation coefficient. A moderated t-test was used for statistical analysis and genes with P-value <0.05 were considered to have significantly different expression.

RESULT AND DISCUSSION

Cell culture and Differentiation

Cell culture was successfully done. At the plating concentration of $2.5x10^4$ cells/well, the cell reached around 90 % confluency in 5 days (Fig 2.1). After induction with induction media the confluent cells started to show accumulation of fat droplets at around 48 hrs. post induction (Fig 2.2). The presence of fat droplets was confirmed using Oil Red O staining, which gave bright red staining to the fat droplets (Fig 2.3).

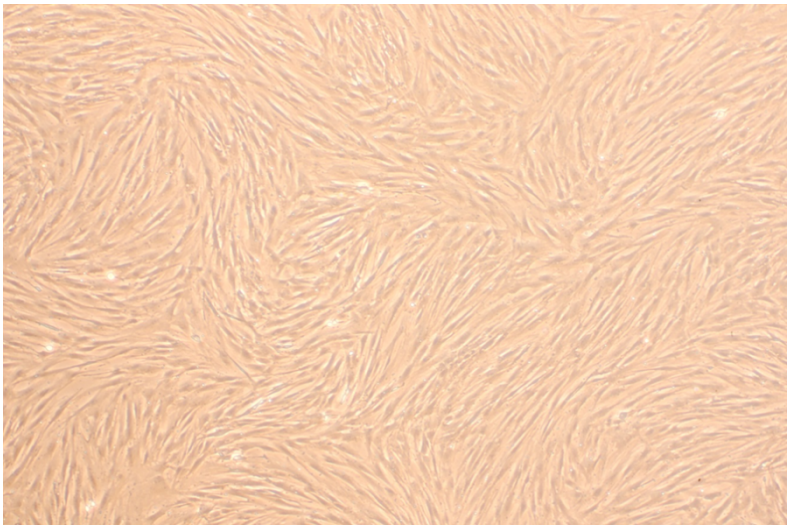
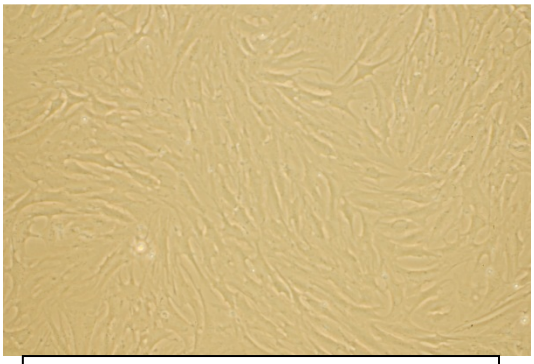
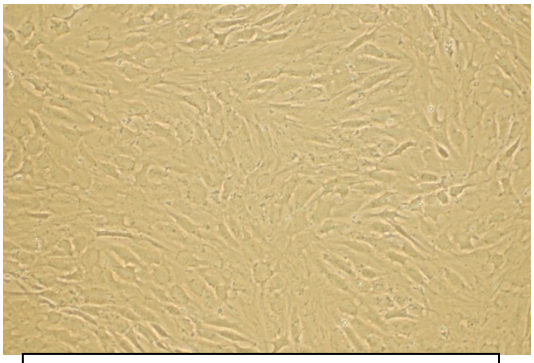


Figure 2.1: Microscopic image of adipocytes at confluency

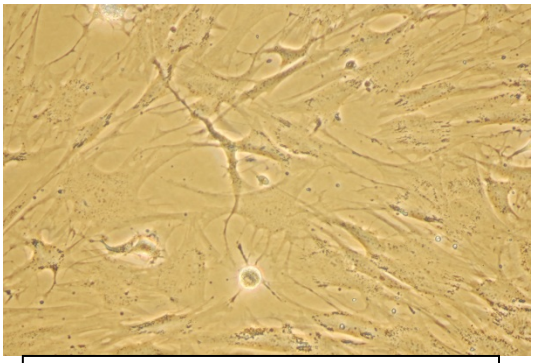
Figure 2.2: Microscopic images of different stages of invitro adipogenesis



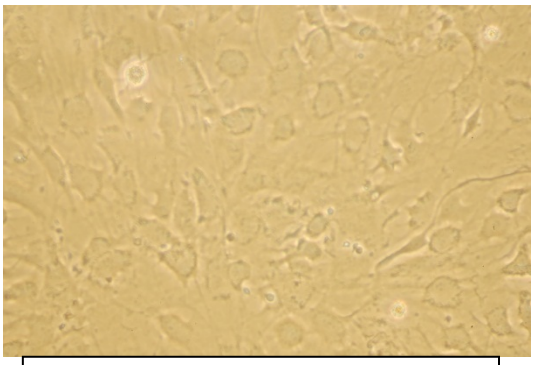
A. Differentiating subcutaneous adipocytes at 12 hr. post induction



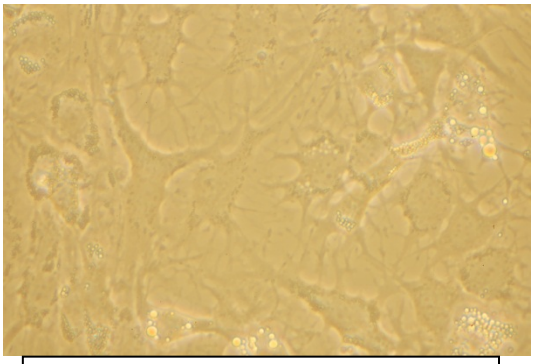
B. Differentiating intramuscular adipocytes at 12 hr. post induction



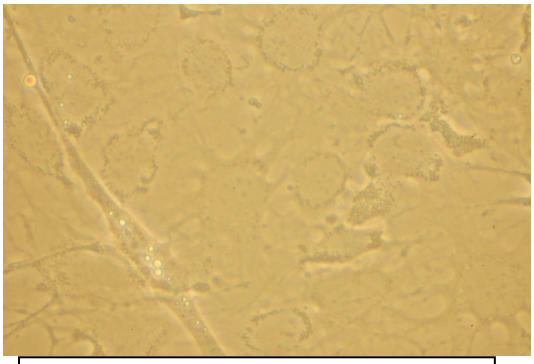
C. Differentiating subcutaneous adipocytes at 24 hr. post induction



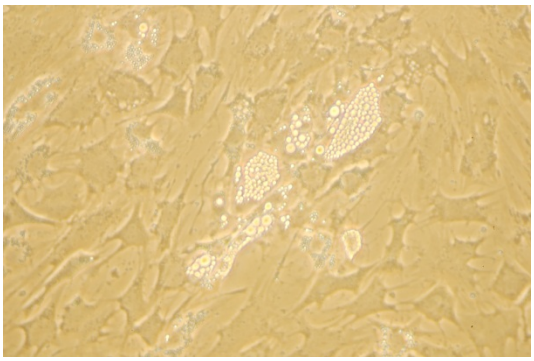
D. Differentiating intramuscular adipocytes at 24 hr. post induction



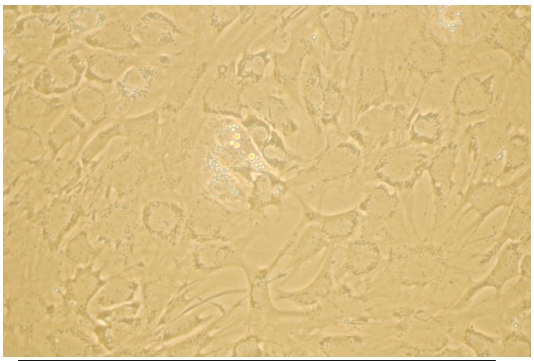
E. Differentiating subcutaneous adipocytes at 48 hr. post induction



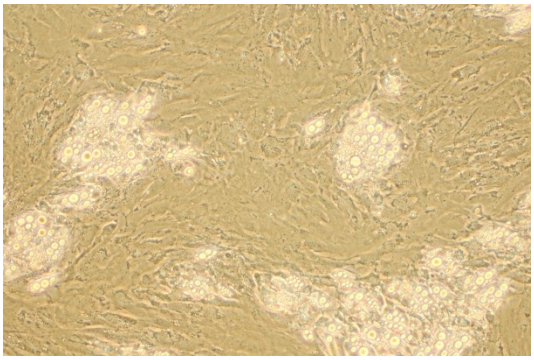
F. Differentiating intramuscular adipocytes at 48 hr. post induction



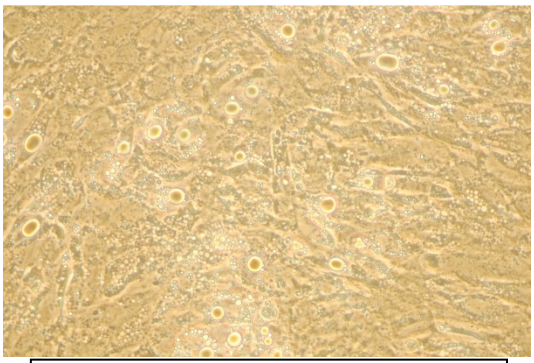
G. Differentiating subcutaneous adipocytes at 72 hr. post induction



H. Differentiating intramuscular adipocytes at 72 hr. post induction



I. Differentiating subcutaneous adipocytes at 8 days post induction



J. Differentiating intramuscular adipocytes at 8 days post induction

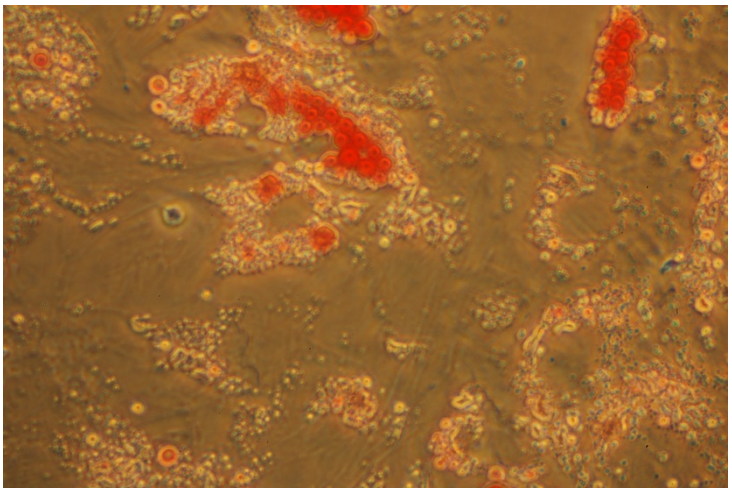


Figure 2.3: Oil Red O stained fat droplets visible in differentiated cell.

Microarray analysis

Microarray study was done using a bovine oligo array containing 24,000 oligonucleotide probes. The processed files were analyzed using GPAP software. The reports generated from GPAP analysis showed that the correlation between the arrays were very low (Table 2.3). Low correlation between the replicates made the data generated by microarray to be statistically insignificant. The hybridized slides of sample from subcutaneous adipocytes on 8 day time point were reanalyzed to check if the correlation can be corrected. For this the slides were rescanned using a more stringent signal threshold value of 3 while defining the outlier as 3 which means that any Log2 ratio value that is outside the 3 standard deviation of the mean. This will incorporate more spots but will effectively filter

out the noisy ones and can improve the correlation between replicates caused due to noisy spots. The resulting correlation did show some improvement but was still very low to be statistically significant. Thus we could not proceed further in our study. Table 2.1 shows the correlation coefficient between replicates before correction and Table 2.2 shows the same values after the correction.

Table2.1: Correlation coefficient between replicate GPR files after pre-processing (Signal threshold=1).

File Name	**Number of Valid Ratio(%)**		
3814 8d SQ.gpr	5977(19.923%)		
4144 8d SQ.gpr	3893(12.977%)		
4248 8d SQ.gpr	5320(17.733%)		
	3814 SQ 8D	**4144 SQ 8D**	**4248 SQ 8D**
3814 SQ 8D	1		
4144 SQ 8D	0.214	1	
4248 SQ 8D	0.1196	0.2696	1

Table 2.2: Correlation coefficient between replicate GPR files after pre-processing (Signal threshold=3).

	Number of Valid Ratio(%)		
3814 SQ 8D	3555(11.850%)		
4144 SQ 8D	3582(11.940%)		
4248 SQ 8D	4575(15.250%)		
Correlation			
	3814 SQ 8D	**4144 SQ 8D**	**4248 SQ 8D**
3814 SQ 8D	1		
4144 SQ 8D	0.4835	1	
4248 SQ 8D	0.5428	0.3846	1

This low correlation amongst the replicates could be due to non-specific binding. This could have occurred during one or more of the several steps of preparation and

hybridization. Non-specific binding can occur due to low temperature of 2X SSC/0.2 SDS during the post hybridization wash. The optimum temperature of the washing solution should be 42ºC. In this study care was taken to maintain the temperature of the washing solution at the optimum temperature by submerging the conical tubes in water bath maintained at 42ºC. The low correlation between arrays coupled with the low number of valid ratio within each array is suggestive of an improper hybridization. This conclusion was based on the fact that the experiment was carried out on the check and proceed basis. The quality of extracted RNA obtained from RNAqueous-4PCR column based kit (Ambion, Austin TX) was checked using the NanoDrop 1000 spectrophotometer. The concentration and amount of dye incorporated in the AA-aRNA was also determined using the NanoDrop 1000 spectrophotometer. Non-specific binding can also occur due to insufficient hybridization time or temperature (Dai et al., 2002). On the other hand excessive temperature during hybridization can cause low signal intensity. The slides were hybridized for 18 hrs. and the temperature in the incubator was set at 42ºC. It can only be speculated that the temperature could have fluctuated during the incubation causing suboptimal hybridization. This could lead to non-specific binding or low signal intensity in some of the slides giving varied expression of genes between the replicates.

Table 2.3: Correlation coefficient between replicate GPR files for all samples.

Sample	**3814 IM 6**	**4144 IM 6**	**4248 IM 6**	**Sample**	**3814 SQ 6**	**4144 SQ 6**	**4248 SQ 6**
3814 IM 6	1			**3814 SQ 6**	1		
4144 IM 6	0.148	1		**4144 SQ 6**	0.1724	1	
4248 IM 6	0.1221	0.1236	1	**4248 SQ 6**	0.1395	0.4401	1
Sample	**3814 IM 12**	**4144 IM 12**	**4248 IM 12**	**Sample**	**3814 SQ 12**	**4144 SQ 12**	**4248 SQ 12**
3814 IM 12	1			**3814 SQ 12**	1		
4144 IM 12	0.1879	1		**4144 SQ 12**	0.2521	1	
4248 IM 12	0.1796	0.1218	1	**4248 SQ 12**	0.3214	0.3925	1
Sample	**3814 IM 24**	**4144 IM 24**	**4248 IM 24**	**Sample**	**3814 SQ 24**	**4144 SQ 24**	**4248 SQ 24**
3814 IM 24	1			**3814 SQ 24**	1		
4144 IM 24	0.1953	1		**4144 SQ 24**	0.1126	1	
4248 IM 24	0.1726	0.2352	1	**4248 SQ 24**	0.2104	0.1218	1
Sample	**3814 IM 48**	**4144 IM 48**	**4248 IM 48**	**Sample**	**3814 SQ 48**	**4144 SQ 48**	**4248 SQ 48**
3814 IM 48	1			**3814 SQ 48**	1		
4144 IM 48	0.221	1		**4144 SQ 48**	0.1462	1	
4248 IM 48	0.1863	0.12	1	**4248 SQ 48**	0.2447	0.2197	1
Sample	**3814 IM 72**	**4144 IM 72**	**4248 IM 72**	**Sample**	**3814 SQ 72**	**4144 SQ 72**	**4248 SQ 72**
3814 IM 72	1			**3814 SQ 72**	1		
4144 IM 72	0.1652	1		**4144 SQ 72**	0.2845	1	
4248 IM 72	0.2459	0.2198	1	**4248 SQ 72**	0.3628	0.2173	1
Sample	**3814 IM 8D**	**4144 IM 8D**	**4248 IM 8D**	**Sample**	**3814 SQ 8D**	**4144 SQ 8D**	**4248 SQ 8D**
3814 IM 8D	1			**3814 SQ 8D**	1		
4144 IM 8D	0.1296	1		**4144 SQ 8D**	0.214	1	
4248 IM 8D	0.1716	0.298	1	**4248 SQ 8D**	0.1196	0.2696	1

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CHAPTER III

REAL TIME ANALYSIS OF GENE EXPRESSION DURING SUBCUTANEOUS AND INTRAMUSCULAR ADIPOGENESIS

INTRODUCTION

Marbling is the deposition of fat in intramuscular depots. It is a desirable trait in beef cattle as it enhances tenderness, texture and palatability of the meat. Therefore marbling influences the quality and economic return of beef carcasses (Cheong et al., 2009; Mizouchi et al., 2010; Pickworth et al., 2011; Sainz, 2000). Although marbling is known to be a late maturing trait, the development of fat starts at an early stage, the level of intramuscular fat in this early stage will help to determine the level of marbling in the adult animal (Lee et al., 2008). Here we study a few genes of interest. These genes were selected for the study based on our prior study done by Vasudevan-Pillai (2008) and from information gained from recent articles on genetic regulation of marbling. The genes in this study are fatty acid binding protein (FABP4), fatty acid translocase (CD36), caveolin 1 (CAV1), phosphatidic acid phosphatase (PPAP2B), phosphoenolpyruvate

carboxykinase 2 (PEPCK) and epithelial membrane protein 3 (EMP3). Phosphatidic acid phosphatase 2B (PPAP2B) regulates phospholipids metabolism and is suggested to be involved in production of PPARγ endogenous ligands. Caveolin 1 was demonstrated to play a critical functional and structural role in the modulation of both lipid droplet biogenesis and metabolism in vivo in a study by Cohen et al. in 2004. The interest in this gene for our study arises from the fact that it was down regulated in our previous study of subcutaneous adipocyte differentiation but was shown to be upregulated in a study done by Mizoguchi et al. using intramuscular adipocytes in 2010. FABP4 is an early marker of adipogenesis, but it has shown to be down regulated during early adipogenesis in bone marrow derived pre-adipocytes (Tan et al., 2006). CD36, a membrane bound glycoprotein, helps in regulating the up-take of long chain fatty acids by adipocytes, heart and muscle cells where long chain fatty acids are important substrate of energy production (Febbraio et al., 2001). PEPCK is a rate limiting enzyme in gluconeogenesis (Caton et al., 2009). A study by Beale et al in 1992, reports that PEPCK mRNA is detectable in 3T3-Ll adipocytes but not in 3T3-Ll fibroblasts. EMP3 is expressed in most tissue with the highest transcripts in peripheral blood leukocytes, ovary, intestine and various embryonic tissues (Taylor et al., 1996). The expression of EMP3 was found to be down regulated during subcutaneous adipogenesis (Vasudevan-Pillai, 2008).

MATERIALS AND METHOD

RNA obtained at the five time points from both subcutaneous and intramuscular cell culture were reverse transcribed using the QuantiScript® Reverse Transcription Kit (QIAGEN Inc., Valencia, CA) using oligo-dT and random primers. RT- PCR reactions were carried using Roche ® SYBR Green Master Mix. The reactions were duplicated for each time point and each tissue and carried out using MyiQ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Gene specific primers for six genes of interest were designed using the NCBI primers designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the primers were analyzed using the Oligo Analyzer 3.1 (Integrated DNA Technologies). The product size and annealing temperature for all primers used are presented in Table 3.1. 18s ribosomal RNA was used for normalization for all genes. Gene expression in the two depots for the selected time points were evaluated using the comparative CT method (Schmittgen and Livak, 2008). A cycle threshold (CT) value for each reaction was obtained at the beginning of the logarithmic phase of the PCR amplification. CT was used to calculated ΔCT value by subtracting it from 18S CT value of corresponding time point. ΔΔ CT was calculated by subtracting the mean for each time point from the mean of control time point '0'. The expression levels of the genes were calculated as fold changes (FC), with each cycle of the PCR representing two-fold change. Calculation for each fold change was done using

the formula FC= 2^-ΔΔ CT for negative ΔΔ CT value and FC= -2^ΔΔ CT for positive ΔΔ CT values (Fernando, B.R, personal communication; Schmittgen and Livak, 2008)

Table 3.1: Primers used for Real time PCR

Primer	Sequence	Size	Tm used	Product size (bp)
FABP4-forward*	AAGCTGCACTTCTTTCTCACC	21	62°C	197bp
FABP4-reverse*	GACCACACCCCCATTCAAAC	20		
CD36-forward*	CAATGGAAAGGACGACATAAG	21	60°C	121bp
CD36-reverse*	TGGAAATGAGGCTGCATCTGT	21		
EMP3-forward*	TCATCCTCTGCTGTCTGTCCTT	22	60°C	159bp
EMP3-reverse*	CCAGAATCTCTTCGGCATGAAT	22		
PPAP2B_forward	GTTATTGCCATCCTCGCGATCATC	24	58°C	285bp
PPAP2B_reverse	TTTGCTGTCTTCGCCTCTGC	20		
PEPCK2_forward	ATCGCCTGGATGAGGTTTGACAG	23	60°C	254bp
PEPCK2_reverse	TTGTCACCAGGTTTCCAGGGTTTG	24		
CAV1_forward	ACGTAGACTCAGAGGGACATCTC	23	58°C	234bp
CAV1_reverse	TGCCATCGAAACTGTGTGTTCC	22		

* Published primers (Vasudevan-Pillai, 2008)

RESULTS

The relative expression level of the genes at four different time points post induction was determined with respect to the control time point i.e. 0 hr. using RT-PCR. The expression level was calculated as mean folds change from the control using the ΔΔCT value. ΔCT

of animal 3814 showed up as more varied from the other two animals used. The variance was only in intramuscular adipocytes and in genes CAV1, FABP4 and PPAP2B. The variance is assumed to be biological as the culture condition for all the biological replicates were maintained constant. Also from the data there is no such variance in ΔCT between animal 3814 and the other two in subcutaneous adipocytes. Hence a Kruskal - wallis non parametric one way ANOVA test was done to check the significance of variance and from it the variance was deemed to be insignificant (P=0.368).

Fatty acid binding protein (FABP4) showed gradual increase in its expression with time. Though the trend was the same in both intramuscular and subcutaneous adipocytes, the level of expression was significantly different with the expression level of the gene in subcutaneous adipocytes as much as 69 times higher than intramuscular (Fig 3.1). Fatty acid translocase (CD36) showed a down regulation of one-folds in subcutaneous adipocytes and 1.72 folds in intramuscular adipocytes at 12 hr. post induction. The gene was upregulated at and after 48 hr. post induction with the highest expression of 70.1 folds up regulation in subcutaneous adipocytes and 17.4 fold increases in intramuscular adipocytes at 72hr post induction in both intramuscular and subcutaneous adipocytes (Fig 3.2). Phosphoenolpyruvate carboxykinase 2 was down regulated at all time points. The amount of down regulation was much higher in intramuscular adipocytes with 12.7 folds down regulation at 48 hr. post induction compared to 2.4 fold down regulation in subcutaneous adipocytes at the same time point (Fig 3.3). Epithelial membrane protein 3 (EMP3) showed a down regulation post induction in both intramuscular and

subcutaneous adipocytes. Similar to PEPCK genes the change in expression was more in intramuscular adipocytes with 95.2 folds down regulation at 48 hr. post induction in intramuscular adipocytes and 28.5 folds down regulation in subcutaneous adipocytes at the same time point(Fig 3.4). Caveolin 1 (CAV1) had a different trend in intramuscular adipocytes than in subcutaneous adipocytes. In subcutaneous adipocytes the expression of CAV1 was shown to be upregulated post induction with the maximum folds change of 16.9 at 48 hr. post induction. Whereas in intramuscular adipocytes the expression was up regulated but the level did not fluctuate highly up to 72 hr. post induction and then at 8 day post induction he expression level of the gene was 9.9 folds down regulated (Fig 3.5). Phosphatidic acid phosphatase 2B (PPAP2B) had very different result in intramuscular and subcutaneous adipocytes. The expression of PPAP2B was upregulated in subcutaneous adipocytes but was gradually down regulated in intramuscular adipocytes. The highest change in expression in subcutaneous adipocytes was 2.9 folds up regulation at 48 hr. post induction. In the intramuscular adipocytes the highest change was 10.6 folds down regulation at 8 days post induction(Fig 3.6). The table below shows the mean folds changes in mean threshold cycle (Ct) and mean standard error for each gene in intramuscular and subcutaneous adipocytes.

Table 3.2: Fold change in expression level of genes in adipocytes at different time points post induction

TISSUE: IM												
	Caveolin IM		**EMP3 IM**		**PPAP IM**		**PEPCK IM**		**CD36 IM**		**FABP4 IM**	
Collected	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**
0 HR	1.00	1.58	1.00	1.49	1.00	1.78	1.00	1.38	1.00	1.72	1.00	1.73
12HR	2.36	1.52	-11.97	1.07	-1.35	1.47	-1.84	1.05	-1.72	1.69	2.12	1.65
48 HR	1.33	1.47	-95.23	1.36	-1.38	1.55	-12.74	1.08	5.49	1.70	1.13	1.50
72 HR	1.14	1.46	-8.62	1.29	-2.62	1.55	-3.41	1.33	17.43	1.57	3.17	1.63
8D	-9.86	2.42	-7.83	2.23	-10.64	3.00	-8.00	2.27	4.21	2.87	2.47	2.81
TISSUE: SQ												
	Caveolin SQ		**EMP3 SQ**		**PPAP SQ**		**PEPCK SQ**		**CD36 SQ**		**FABP4 SQ**	
Collected	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**	**MFC**	**Error-FC**
0 HR	1.00	1.25	1.00	1.17	1.00	1.30	1.00	1.16	1.00	1.61	1.00	1.30
12HR	4.21	1.26	-7.59	1.32	1.00	1.16	-1.55	1.27	-1.01	1.30	13.69	1.38
48 HR	16.87	1.46	-28.57	1.29	2.87	1.33	-2.04	1.53	30.70	1.51	69.23	1.49
72 HR	8.51	1.20	-6.13	1.09	2.46	1.39	-2.37	1.17	70.12	1.35	95.34	1.28
8D	1.53	1.21	-1.89	1.18	1.15	1.12	-1.09	1.26	58.01	1.16	49.64	1.14

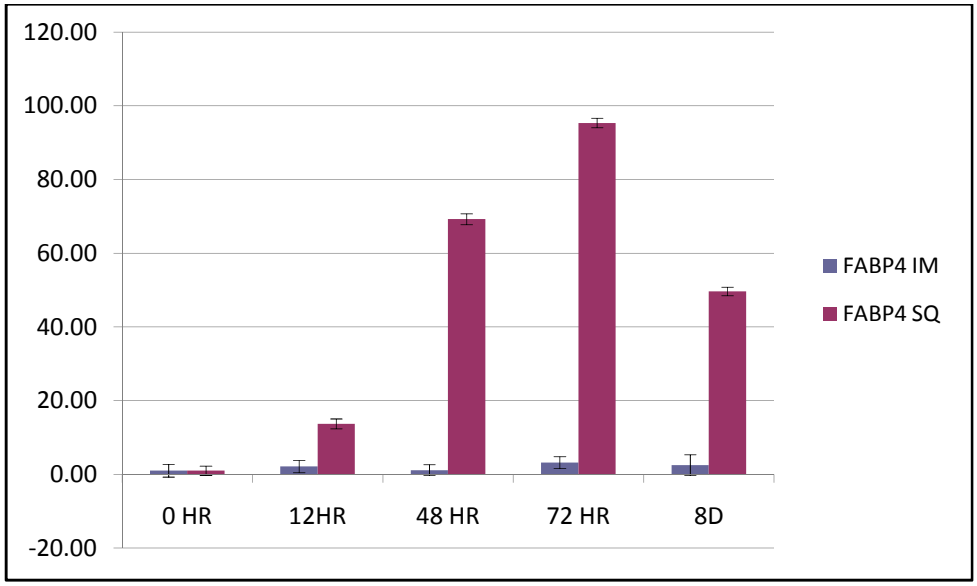

Figure 3.1: Graphical representation of relative changes in expression of FABP4 post induction in intramuscular and subcutaneous adipocytes.

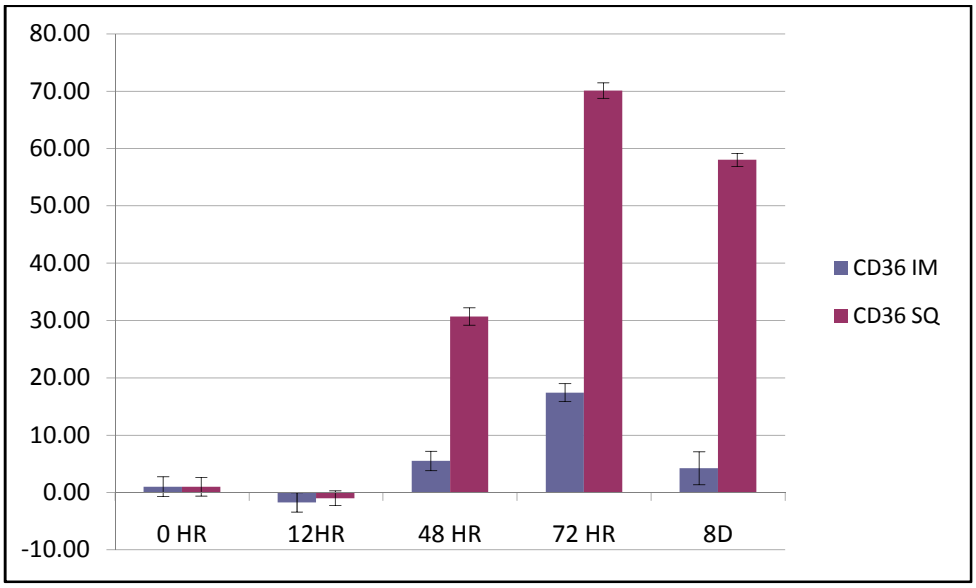

Figure 3.2: Graphical representation of relative changes in expression of CD36 post induction in intramuscular and subcutaneous adipocytes.

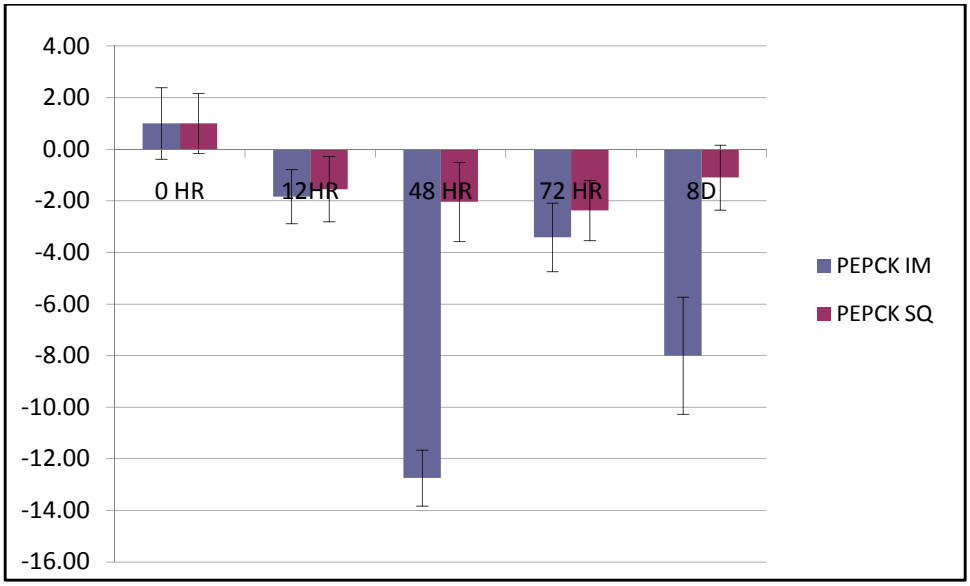

Figure 3.3: Graphical representation of relative changes in expression of PEPCK post induction in intramuscular and subcutaneous adipocytes.

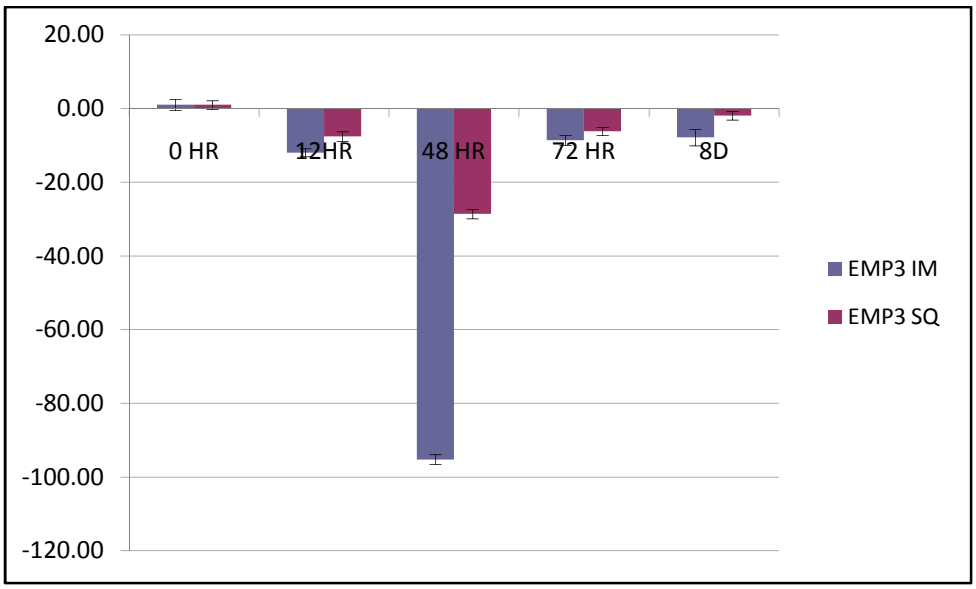

Figure 3.4: Graphical representation of relative changes in expression of EMP3 post induction in intramuscular and subcutaneous adipocytes.

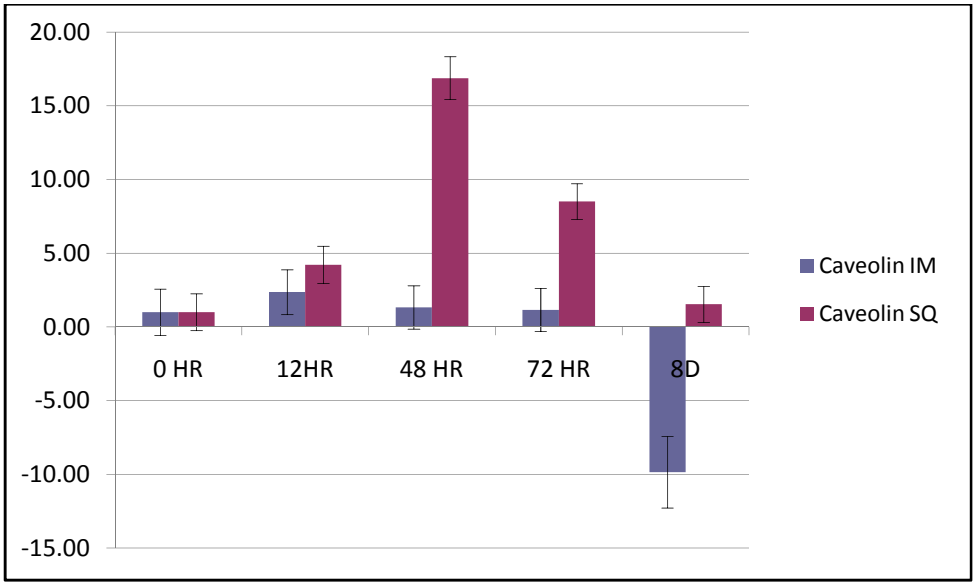

Figure 3.5: Graphical representation of relative changes in expression of PPAP2B post induction in intramuscular and subcutaneous adipocytes.

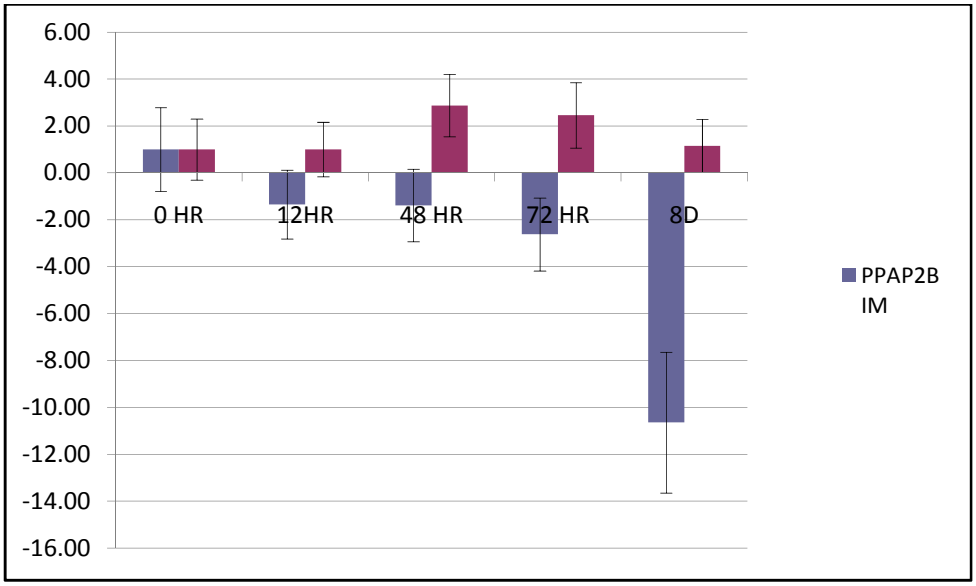

Figure 3.6: Graphical representation of relative changes in expression of PEPCK post induction in intramuscular and subcutaneous adipocytes.

DISCUSSIONS

Intramuscular adipocytes arise from the mesenchymal stem cells (MSCs) which are very abundant in the skeletal muscle at the early stages of development. Most of the MSCs will develop into myogenic cells and only a small fraction of it will develop into adipogenic cell, which results intramuscular fat accumulation (Du et al., 2009). Marbling in cattle is governed by its genetic makeup, nutrition and environmental factor. Nutrition is a key factor in increasing marbling (Tong et al., 2009). The problem with such an approach is that for each pound of intramuscular fat obtained there is a 10 pound increase in waste fat (H. Rouse, 2001). Genetic makeup of an animal is very important because an animal can be provided a very suitable environment and a balanced diet but unless it has the right genetic makeup, marbling will not be higher compared to an animal on a regular diet and with the right genetic makeup for high marbling (Wang et al., 2005). Though there has been a steady development in identifying the markers for marbling, most of the information has been drawn from rodent (3T3-L1) and human cell culture system. It has also been shown that the expression of pattern of some key regulators of adipogenesis differs significantly in bovine intramuscular preadipocytes from the 3T3 cell lines (Mizoguchi et al., 2010). It has also been shown that the genes expression during intramuscular and subcutaneous adipocytes differ in various studies (Mizoguchi et al., 2010). Hence this study comparing the gene expression during intramuscular adipogenesis and subcutaneous adipogenesis in respect to 6 different genes will help understand their importance in marbling.

Fatty acid binding proteins (FABPs) are a family of conserved intracellular lipid binding proteins. FABP 4 is an adipocyte-derived fatty acid binding protein and it functions to enhance the transcriptional activity of PPARγ. FABP4 is an early adipogenic marker. The expression of FABP4 was up regulated in both intramuscular and subcutaneous adipocytes in our study, which conforms to various other studies on FABP4 (Urs et al., 2004; Mizoguchi et al., 2010)

Fatty acid translocase (CD36) in membrane glycoprotein expressed in many tissues and cell types, including the adipocytes. It is an important player in the uptake of long chain fatty acid and is responsible for the uptake and oxidation of fatty acids in skeletal muscle and lipid accumulation in adipocytes and recently it has been established that Cd36 also functions in the degradation of oxidized low density lipoprotein (Kuniyasu et al., 2002; Qioa et al., 2008). In our study CD36 was upregulated in both adipocytes except for the time point 12 hr. post induction. The up regulation of the gene during adipogenesis is in confirmation with other such studies done in mouse 3T3 cells (Kuniyasu et al., 2002; Sun et al., 2003). The down regulation of the gene at 12 hr. post induction can be explained by the presence of IBMX and Dexamethasone in the induction media I. In a 2003 study done by Sun et al comparing the expression of Cd36 in response to adipogenic agents in macrophages and mouse 3T3 cell, it is shown that the expression level of CD36 in macrophages is down regulated in the presence of adipogenic agents IBMX and Dexamethasone. In the same study it has been shown that the expression of Cd36 is increased in presence of IBMX and Dexamethasone together with Insulin. The presented data is at 4 days post induction and it fails to clarify whether the induction media was

changed at all during that period. The expression level of gene could have been influenced by the fact that the effect of dexamethasone and IBMX could have weakened with time. In our study the induction media was changed every 48 hr. and in the Induction media II both Dexamethasone and IBMX were left out, which can explain the up regulation of the gene at and after 48 hr. post induction.

Caveolins are a conserved family of membrane-associated proteins from three genes that have tissue specific expression. Caveolin-1 and 2 are present in endothelial cells, type-II pneumocytes and adipocytes whereas caveolin-3 is only present in muscles. Adipocyte derived Caveolin-1(CAV1) plays a role in caveolae formation (Scherer al., 1994). Caveolae are specialized lipid rafts present in the plasma membrane. It functions in membrane traffic, signal transduction, substrate transport, lipid storage and global fat cell function (Briand et al., 2011; González-Muñoz et al., 2009). In our study CAV1 showed a rise and fall trend with 16 fold increase in expression at 48 hr. post induction falling down to 1.5 fold up regulation at 8 days post induction in subcutaneous adipocytes. The expression of CAV1 in intramuscular adipocytes was highest with 2 fold increase at12 hr. The expression of the genes was down regulated 9.86 folds at 8 days post induction in intramuscular adipocytes. In the previous study by Vasudevan-Pillai (2008) in subcutaneous adipocytes using microarray analysis CAV1 was shown to be upregulated which confirms with our real time analysis. Interestingly a study done by Mizoguchi et al in 2010 using serial gene analysis shows the expression of CAV1 to be down regulated in intramuscular adipocytes. Mizoguchi et al. studied the genes expression 4 days post

induction hence it can be speculated that the expression of CAV1 starts to decrease after 3 days post induction as our data shows it to have up regulated up until that point in intramuscular adipocytes. It can be speculated from the above information that CAV1 is necessary for the differentiation process but not to maintain the differentiated state. There are however some contrasting data on CAV1 expression during adipogenesis. One of which is a study done by Takenouchi et al also using BIP cell lines. Their study shows a gradual up regulation of CAV1 from 0 to 8 days post induction. A 2004 study by Park et al., overexpression of CAV1 is associated an inhibitory effect of CAV1 on adipogenic differentiation in young hMSCs. This shows that a further study on this gene is necessary to determine the detailed role of CAV1 in adipogenesis.

In our study the Expression of PEPCK2 was down regulated in both intramuscular and subcutaneous adipocytes. Phosphoenolpyruvate carboxykinase (PEPCK) is found in tissues, including the liver, kidney cortex, and brown and white adipose tissue (Nye et al., 2008). PEPCK occurs in two isoforms, cytosolic (PEPCK-C/PEPCK1) and mitochondrial (PEPCK-M/PEPCK2). PEPCK1 is encoded by PCK1 gene and PEPCK2 by PCK2 gene. PEPCKs also play a crucial role in glyceroneogenesis (Beale et al., 2007; Nye et al., 2008). PEPCK1 has been extensively studied but PEPCK2 has been ignored. This is mostly because the isoforms are tissue and species specific with PEPCK1:PEPCK2 ration being 95:5 in rats and 90:10 in birds and 50:50 in human (Beale et al., 2007; Nye et al., 2008). Since there is limited information available on PEPCK2 in cattle, further studies can be done to find its function and importance in cattle.

Epithelial membrane protein (EMP3) expression conformed with prior study done on subcutaneous adipocytes. The results show that the expression of EMP3 is consistent during subcutaneous and intramuscular adipogenesis.

Phosphatidic acid phosphatases (PPAP) are small integral membrane glycoproteins. It functions in synthesis of lipids and also in generation/degradation of lipid-signaling molecules in eukaryotic cell. PPAP is a rate limiting enzyme in triglyceride synthesis and is influenced highly by nutrition and hormonal status (Coleman et al., 2000). PPAP functions as a catalyst for phosphorylation of phoshphatidate to diacylglycerol and inorganic phosphate (Carman et al., 2006). PPAP has been reported to have higher expression in bovine subcutaneous preadipocytes than in intramuscular preadipocytes by Smith et al in 1998. In the same study it was found that the level of PPAP did not change in intramuscular preadipocytes during starvation but was drastically down regulated in the subcutaneous preadipocytes (Oritz-Colon., 2006). Two forms of PPAP were recently identified based on their subcellular localization and function. PPAP1 is a cytosolic form and its catalytic activity is Mg2+ dependent. PPAP2 is an Mg2+ independent catalyst, and the better studied of the two (Sciorra et al, 1999). Kai et al in 1997 reported two isoforms of PPAP2, PPAP2A and PPAP2B. Recently a third isoform PPAP2C has also been identified (Hooks et al., 1998). PPAP2B has been studied for its role in metabolism of phosphatidic acid derived from the activation of Phosphatidycholine-specific phospholipase D (PLD) producing diacylglycerol by Sciorra et al., in 1999. Their study has established the presence of PPAP2B in caveolin-1 enriched detergent resistant

membrane micro domains. This is in line with our findings; the expression of Caveolin-1 is down regulated along with the expression of PPAP2B in our study.

CONCLUSION

The present study gives comparative changes in gene expression between intramuscular and subcutaneous adipogenesis in respect to six different genes. While 4 out of the six genes showed a similar trend in both preadipocytes, the level of expression differed considerably with intramuscular preadipocytes showing a lower expression of those genes. This difference in expression level can be attributed to the lower percentage of differentiation of intramuscular preadipocytes compared to subcutaneous preadipocytes (Fig 2.1). The difference in expression pattern of CAV1 gene which is an important player in lipid storage is interesting. Our study shows a decline in CAV1 expression in intramuscular adipocytes on 8 days post induction. Based on the expression of adipogenic marker genes FABP4 and CD36 in intramuscular adipocytes it is possible that the limiting factor for marbling is not adipogenesis per se but the lack of storage ability in the depot combined with decreased adipogenesis. The down regulation of PPAP2B which is a rate limiting enzyme in triglyceride synthesis further supports this hypothesis. Thus it is possible that the numbers of adipocytes in intramuscular depots are less compared to subcutaneous depots resulting in lower marbling, which is further effected by the intramuscular adipocytes lack of ability to synthesize and store lipids. Thus it can be

concluded that a detailed study on CAV1 and PPAP2B will generate more insight as to the genetic impact on marbling of beef cattle.

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Title of Study: **DIFFERENTIAL GENE EXPRESSION DURING ADIPOGENESIS IN CULTURED BOVINE ADIPOCYTES**

Pages in Study: 77 Candidate for the Degree of Master of Science

Major Field: Animal Science

Scope and Method of Study:

Gene expression during adipogenesis was studied using Real time PCR in cultured intramuscular and subcutaneous bovine adipocytes. The study was conducted to understand expression changes in, Fatty acid binding protein 4(FABP4),Fatty acid translocase(CD36), Epithelial membrane protein(EMP3),Phosphoenol pyruvate carboxykinase 2(PEPCK2),Caveolin 1(CAV1) and Phosphatidic acid phosphatase(PPAP2B) during intramuscular (i.m) and subcutaneous(s.c) adipogenesis. The study consisted of 3 biological replicates and the differential gene expression was studied at 12 hr., 48 hr., 72 hr. and 8 days post induction. The gene expression study was done using real time PCR analysis using ribosomal 18s gene to normalize all genes and changes in gene expression was presented as fold change from the expression at control time point i.e. 0 hr. post induction.

Findings and Conclusions:

FABP4, CD36, EMP3 and PEPCK2 showed a similar expression pattern in both adipocytes. However the amount of expression was very low in i.m adipocytes for the genes compared to s.c adipocytes. This can be attributed to lower differentiation of i.m preadipocytes. CAV1 and PPAP2B were differently expressed in the two adipocytes. CAV1 sharply down regulated in i.m adipocytes on 8 days post induction, and PPAP2B showed down regulation in i.m adipocytes from 12 hr. post induction while both the genes were upregulated in s.c adipocytes. From our finding that CAV1 which plays a role in lipid storage and PPAP2B which plays a role in triglyceride synthesis were down regulated in i.m adipocytes but other adipogenic genes are expressed similarly to s.c adipocytes we can conclude that the cause of less accumulation of fat in intramuscular depot might not only due to lesser adipogenesis but also to decreased ability of i.m adipocytes to synthesize and store lipids.

ADVISER'S APPROVAL: Dr. Udaya DeSilva